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Epidermal Growth Factor Stimulates Ornithine Decarboxylase Activity in Cultured Mammalian Keratinocytes*

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The influence of epidermal growth factor (EGF) on ornithine decarboxylase has been examined in cultured bovine keratinocytes. Keratinocyte ornithine decarboxylase activity was maximal at pH 6.3 in MES buffer in the presence of dithiothreitol and EDTA. When cultured cells, deprived of serum, were exposed to EGF, the activity of ornithine decarboxylase was stimulated severalfold. Enzyme activity increased in a dose-dependent manner with EGF. The time course of this stimulation is unlike any previously reported in cultured cells. The increase in activity was maximal by 8 h. A small dip in activity was seen between 8 and 12 h. Increased activity was sustained for as long as 24 h after exposure to EGF. The prolonged increase in enzyme activity was reduced by actinomycin D. When cycloheximide was added 1 h before EGF, ornithine decarboxylase activity was obliterated.

This is the first demonstration of ornithine decarboxylase stimulation following exposure to EGF in cultured keratinocytes. The prolonged duration of ornithine decarboxylase stimulation is unexplained but may be related to processing of EGF by the keratinocytes.

Ornithine decarboxylase (L-ornithine carboxy-lyase, E.C. 4.1.1.17) catalyses the decarboxylation of ornithine to putrescine, an important step in polyamine biosynthesis [1]. Ornithine decarboxylase activity increases in response to a variety of agents which induce cell growth and proliferation [1]. In mouse skin exposed to the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), ornithine decarboxylase activity increases within 4 h [2]. Within 1 day mouse epidermis thickens in response to TPA [3].

Epidermal growth factor (EGF), a peptide of molecular weight 6045 isolated from mouse submaxillary gland [4], has been shown to produce epidermal thickening in animals [5] and in organ culture [6]. Ornithine decarboxylase activity is increased when mouse skin is injected with EGF and when organ cultures of chick embryo skin are exposed to EGF [7]. In both instances the increase in ornithine decarboxylase activity precedes the epidermal thickening. EGF produces an increase in ornithine decarboxylase activity in cultured fibroblasts [8], granulosa cells [9], kidney cells [10], and rat hepatoma cells [11]. EGF alters the distribution of keratin filaments in cultured mouse embryonic epithelial cells [12]. EGF also facilitates the culture of normal human keratinocytes:† the number of cells produced in culture, the number of generations of cells produced, and the plating efficiency of the cultured cells are enhanced by EGF [13,14]. Despite this biologic response to EGF, no biochemical response to EGF has been reported in normal cultured keratinocytes.

Because of the effects of EGF on ornithine decarboxylase activity in mouse skin and in cultured cells and because of the biologic effects of EGF on mouse skin and on cultured human keratinocytes, we studied the response of ornithine decarboxylase activity to EGF in cultured mammalian epithelial cells. We studied the effect of EGF on ornithine decarboxylase activity after determining optimal conditions for measurement of ornithine decarboxylase activity. Here we report that EGF stimulates ornithine decarboxylase activity in cultures of bovine keratinocytes.

MATERIALS AND METHODS

Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin solution, Dulbecco's phosphate-buffered saline (PBS), and 0.25% (1/250) trypsin solution were obtained from GIBCO Laboratories, Grand Island, New York. Fetal bovine serum and calf serum were obtained from GIBCO or from Flow Laboratories, Rockville, Maryland. Gentamicin reagent solution was obtained from Schering Corporation, Kenilworth, New Jersey. Mouse embryonic fibroblasts, CCL-92 (3T3 cells) were obtained from the American Type Culture Collection, Rockville, Maryland. ACES, actinomycin D, ADA, cholamine chloride, dithiothreitol, EDTA (free acid), HEPES, MES, L-ornithine, PIPES, pyridoxal phosphate, and TRIS were obtained from Sigma Chemical Co., St. Louis, Missouri D,L-[1-^{14}C]ornithine, 30–55 mCi/mmol, [methyl-^{3}H]thymidine (2 Ci/mmol), Hydrofluor, and Liquifluor were obtained from New England Nuclear, Boston, Massachusetts. All other reagents were of reagent grade and were obtained from commercial sources.

Cell Culture

Keratinocytes were cultured according to the method of Rheinwald and Green [15], as modified by Milstone et al [16]. Bovine esophageal foreskin epithelium was separated from underlying connective tissue by incubation in trypsin. Keratinocytes were dissociated mechanically and added to dishes containing previously irradiated 3T3 cells. Medium contained DMEM, 20% fetal bovine serum, 0.4 μg/ml hydrocortisone, and 100 U penicillin–100 μg streptomycin/ml solution, and gentamycin, 50 μg/ml. Gentamicin was omitted beginning with the first medium change. Medium was changed twice weekly; cultures were maintained at 37°C in a humidified atmosphere of air and 5% CO_2. All experiments were performed on confluent stratified primary calf esophageal keratinocyte cultures 4–12 weeks after plating.

Epidermal Growth Factor

EGF was purified from mouse submaxillary glands [17]. Aliquots were stored in 0.1% bovine serum albumin in Ca^{2+},Mg^{2+}-free PBS at

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Abbreviations:
ACES: N-[2-acetamido]-2-aminoethanesulfonic acid
ADA: N-[2-acetamido]-2-iminodiacetic acid
cholamine chloride: 2-aminoethyltrimethylammonium chloride
DMEM: Dulbecco's modified Eagle medium
EGF: epidermal growth factor
MES: 2-[N-morpholino]ethanesulfonic acid
PBS: phosphate-buffered saline
PIPES: piperazine-N, N′-bis[2-ethansulfonic acid]
3T3: mouse lung embryonic fibroblasts, CCL-92
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

† In this paper keratinocytes are defined as epithelial cells that make the keratin proteins.

−70°C. The EGF was pure by amino acid analysis and had biologic activity when injected into newborn mice. When rabbit anti-EGF antiserum (Collaborative Research, Waltham, Massachusetts) was reacted against EGF on Ochterlony gel, a line of identity was formed; no reaction was formed against preimmune rabbit serum.

Ornithine Decarboxylase Activity

Ornithine decarboxylase activity was determined by measuring $^{14}CO_2$ enzymatically released from L-(1-^{14}C)ornithine [18]. In a typical experiment cultures were given DMEM containing 20% fetal bovine serum for 4 days. They were then washed 3 times with Ca^{2+},Mg^{2+}-free PBS and given serum-free DMEM containing no antibiotics or hydrocortisone. Cultures were incubated 24 h and either EGF or vehicle (Ca^{2+},Mg^{2+}-free PBS with 0.1% bovine serum albumin) was added in a small volume. Cultures were incubated an additional 10 h and then placed on ice. The medium was removed, cultures were washed 3 times with cold Ca^{2+},Mg^{2+}-free PBS, and harvested in 500 μl Ca^{2+},Mg^{2+}-free PBS by scraping from the culture dish with a silicone policeman. The harvested cells were centrifuged at 4°C, 6400 *g* for 10 min. The pelleted cells were resuspended in 300 μl of reaction buffer. Unless otherwise stated the final reaction mixture contained 50 mM MES (pH 6.3 at 37°C), 0.1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM dithiothreitol in 100 μl final volume. Resuspended cells were frozen in liquid nitrogen 45 s and thawed in water at room temperature for 3 cycles. The broken cell suspension was centrifuged and 90 μl of the supernatant was placed in a 17 × 100 mm polystyrene disposable culture tube and preincubated at 37°C for 10 min. The reaction was initiated by the addition of 10 μl of substrate which consisted of D,L-[1-^{14}C]ornithine and unlabeled L-ornithine. The final specific activity of the L-ornithine was 4.5 mCi/mmol. Each reaction mixture contained 0.55 mM L-ornithine (0.25 μCi). The culture tube was capped with a serum stopper from which was suspended a polypropylene center well (Kontes, Vineland, New Jersey) containing 200 μl NCS (Amersham, Arlington Heights, Illinois). The tube was placed in a shaking water bath incubator at 37°C for 1 h. The reaction was stopped by injecting 0.5 ml 5 N sulfuric acid through the serum stopper and the incubation was continued for 1 more hour. The center well containing NCS was placed in a scintillation vial, 5 ml Hydrofluor was added, and the samples were counted in a Beckman LS 7000 liquid scintillation counter at 4°C. Blank values, obtained by incubation of isotope in assay buffer only, were subtracted. Data were corrected to dpm with the use of an external standard and are expressed as pmol ornithine decarboxylated per mg protein per hour (pmol mg^{-1} hr^{-1}). Protein was determined by a modification of the Lowry method [19] using bovine serum albumin as a standard.

Thymidine Incorporation

Cultures were incubated in serum-free DMEM for 24 h; then either EGF or vehicle was added or medium was exchanged for fresh DMEM containing 20% fetal bovine serum. Several hours later, [^{3}H]thymidine (1 μCi, 0.5 nmol/ml medium) was added for 2 h. Dishes were then washed 3 times with cold Ca^{2+},Mg^{2+}-free PBS and 1 ml cold 5% trichloroacetic acid was added. The dishes were placed on ice for 20 min; the cultures were then harvested by scraping into the cold 5% trichloroacetic acid. Harvested material was transferred into 10 × 75 mm culture tubes. Dishes were rinsed with an additional 0.5 ml cold 5% tricholoracetic acid. The harvested material was centrifuged at 4°C, 2300 *g*, for 10 min and the pellet was washed twice with 1 ml cold 5% tricholoracetic acid. Five hundred microliters of 0.5 N NaOH was added and the pellet solubilized by heating at 100°C for 15 min. Radioactivity in the solubilized material was determined with a modification of the method of Mans and Novelli [20]. Aliquots were spotted on Whatman 3MM filter paper discs 2.4 cm in diameter and pierced with brass dressmakers' pins. The discs were washed once in 10% tricholoroacetic acid for 30 min at 4°C, then 4 times with 5% tricholoroaetic acid for 15 min at 4°C. The discs were then washed with ethanol:ether (1 vol:3 vol) for 15 min at room temperature and ether for 15 min at room temperature. Discs were air dried, placed in polyethylene scintillation vials with 5 ml Liquiflour, and counted. Specific activity is expressed as dpm/mg protein.

RESULTS

Cultures

Keratinocytes form colonies and divide; at 3 weeks the cultures form a stratified epithelium. Cell renewal and cell loss from the cultures are roughly equal and the stratified cultures maintain a constant appearance for the following 2–3 months [16].

Ornithine Decarboxylase—Assay Conditions

Three to four days after the addition of fresh medium to cultures, ornithine decarboxylase activity was maximal in MES buffer pH 6.3 in the presence of pyridoxal phosphate, dithiothreitol, and EDTA. At a given pH, the activity of ornithine decarboxylase depended on the buffer used; at pH 6.9 ornithine decarboxylase activity was greater in MES than in 6 other buffers (Table I). Phosphate ion (50 mM) inhibited enzyme activity by approximately 50% when compared with TRIS-HCl (57 vs 38 pmol mg^{-1} hr^{-1} at pH 7.1). No activity was detectable in TRIS-maleate (pH range 5.0–8.5). A broad pH optimum with a maximum of 6.3 was seen in MES (Fig 1).

Enzyme activity was stabilized by dithiothreitol, EDTA, and pyridoxal phosphate, and was stable at −70°C for at least 4 days. Enzyme activity was linear as a function of time for at least 70 min and as a function of protein concentration up to 540 μg protein/90 μl assayed. Pyridoxal phosphate was required; ornithine decarboxylase activity was 64 and 320 pmol mg^{-1} hr^{-1} without and with the cofactor. Apparent K_m of the enzyme for ornithine was 61 μM, and for pyridoxal phosphate was approx-

TABLE I. *Effect of buffer on ornithine decarboxylase activity*

Buffer	Ornithine decarboxylase activity (pmole mg^{-1} hr^{-1})
MES	486 ± 23
TRIS	384 ± 33
PIPES	337 ± 24
ACES	310 ± 46
HEPES	298 ± 24
Cholamine chloride	141 ± 1
ADA	92 ± 28

Four days after medium change, bovine keratinocytes were harvested in each of the above buffers and ornithine decarboxylase activity was determined as described in *Materials and Methods*. Each final reaction mixture contained 50 mM buffer, 0.1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM dithiothreitol in 100 μl final volume. All buffers were adjusted to pH 6.9 at 37°C after the addition of EDTA. Results are expressed as mean ± SE of duplicate assays from 1 culture.

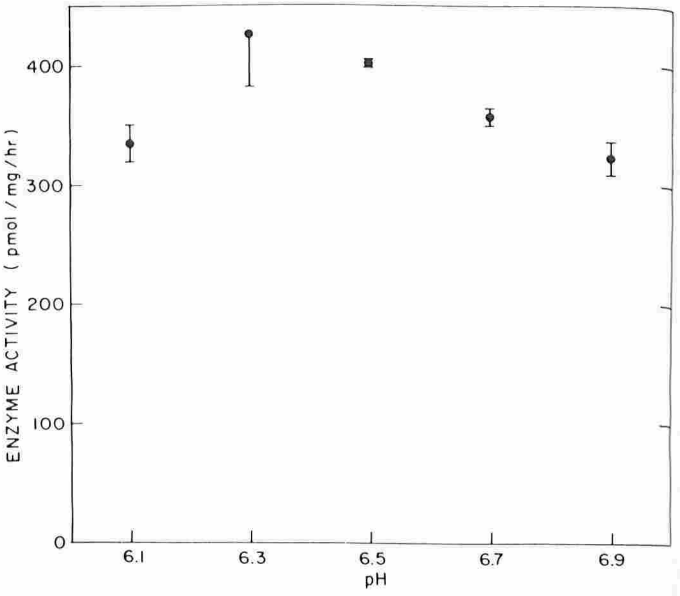

FIG 1. Ornithine decarboxylase activity vs pH. Four days after medium change, bovine keratinocytes were harvested in extraction buffer of varying pH and ornithine decarboxylase activity was determined as described in *Materials and Methods*. Each point represents the mean ± SE of material combined from 2 cultures and assayed in triplicate.

imately 0.13 μM. Recovery of added [^{14}C]bicarbonate as $^{14}CO_2$ was 94%.

Epidermal Growth Factor Effects on Ornithine Decarboxylase Activity

Replacement of medium with fresh serum-free DMEM stimulated ornithine decarboxylase activity (Fig 2). Cultures were given fresh complete medium; 3–4 days later they were washed 3 times with Ca^{+2},Mg^{+2}-free PBS and serum-free DMEM was added. Ornithine decarboxylase activity increased to a maximum (6- to 8-fold) 4 h after the medium change and then rapidly declined. At 15 and 24 h after medium change ornithine decarboxylase levels were approximately the same as those seen before manipulation.

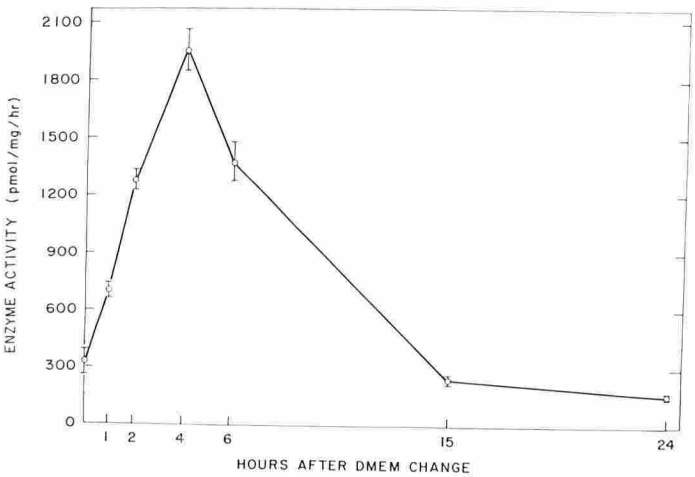

FIG 2. Ornithine decarboxylase activity after DMEM change vs time. Three days after medium change, bovine keratinocytes were washed 3 times with Ca^{2+},Mg^{2+}-free PBS, given serum-free DMEM, and incubated at 37°C. Cells were harvested at the times indicated and assayed for enzyme activity as described in *Materials and Methods*. Each point indicates the mean ± SE of 2 dishes, each assayed in duplicate.

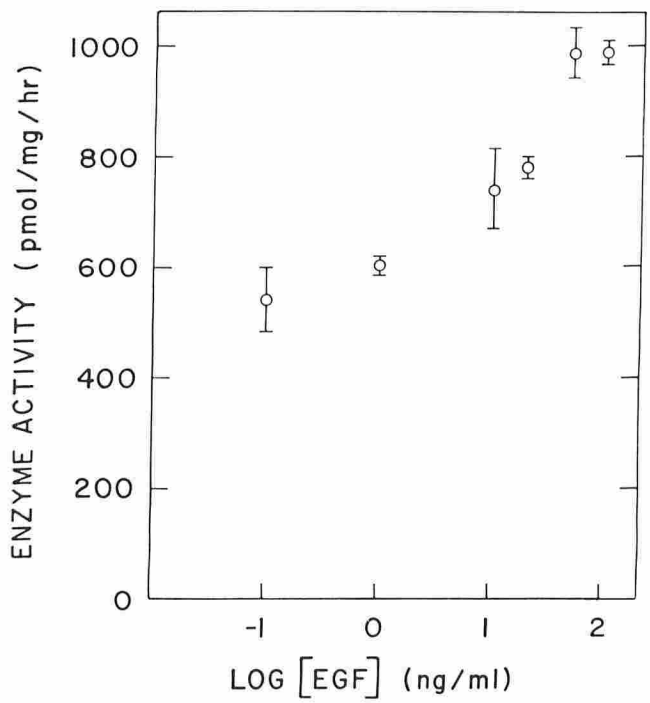

FIG 3. Ornithine decarboxylase activity vs EGF concentration. Three days after medium change, bovine keratinocytes were washed 3 times with Ca^{2+},Mg^{2+}-free PBS and given serum-free DMEM. Twenty-four hours later cultures were exposed to EGF (concentration range from 0.1 ng/ml to 100 ng/ml – 1.65×10^{-11} M to 1.65×10^{-8} M) for 11 h, harvested, and assayed for enzyme activity as described in *Materials and Methods*. Each point indicates the mean ± SE of duplicates from 2 dishes.

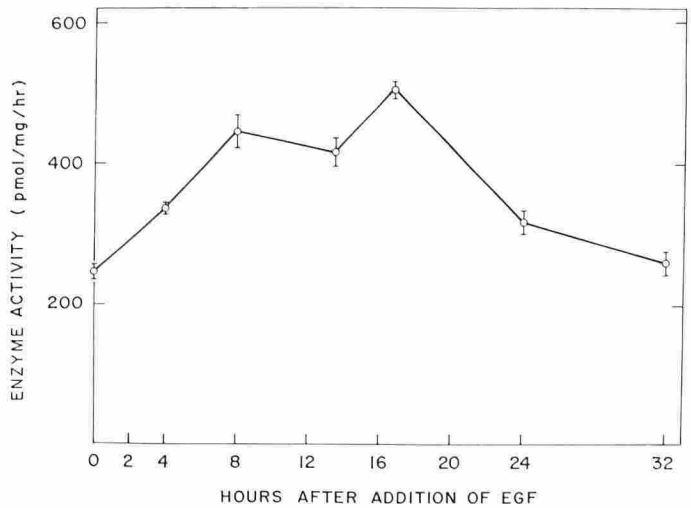

FIG 4. Time course of EGF effect on ornithine decarboxylase. Bovine keratinocytes were exposed to 50 ng/ml (8.27×10^{-9} M) EGF as described in Fig 3. At the times indicated, cells were harvested and ornithine decarboxylase activity was determined as described in *Materials and Methods*. Each point represents the mean ± SE of duplicates from 2 dishes.

TABLE II. *Effect of EGF on [^{3}H]thymidine incorporation*

Addition to culture	Time after addition		
	16 h	20 h	21.5 h
EGF	2134	1970 ± 45	1668 ± 57
Vehicle	2337 ± 362	1779	1622 ± 110
Re-feed	9855	8662 ± 1190	7999 ± 1780

Four days after feeding, bovine keratinocyte cultures were washed 3 times with Ca^{2+},Mg^{2+}-free PBS and serum-free DMEM was added. Twenty-four hours after addition of serum-free DMEM, EGF or vehicle was added or the cultures were re-fed with DMEM containing 20% fetal calf serum. [^{3}H]Thymidine (1 μCi and 0.5 nmol/ml medium) was added at times after addition of EGF, and trichloroacetic acid-precipitable counts were determined after a 2-h pulse, as described in *Materials and Methods*. Results are expressed as the mean dpm/mg protein ± SE of the cultures, each assayed in duplicate.

Removal of cultures from the incubator and addition of vehicle had no effect on ornithine decarboxylase activity. Adding EGF to cultures 24 h after changing to serum-free DMEM produced an increase in ornithine decarboxylase activity. The increase in enzyme activity was related to the concentration of EGF; ornithine decarboxylase activity reached maximal levels when the concentration of EGF was 50 ng/ml medium—approximately 8.3 nM (Fig 3). The ornithine decarboxylase response to EGF differed from the response to serum-free medium in two ways: the maximal increase was less with EGF and the duration of response to EGF was much longer (Fig 4). The increase in ornithine decarboxylase activity in response to EGF reached a maximum (2- to 3-fold) approximately 8 h after addition of EGF. This increased activity persisted with a small dip between 8–16 h. Enzyme activity began to decrease approximately 16 h after addition of EGF, approaching original levels by 32 h after addition of EGF. The bimodal curve shown in Fig 4 was seen in 3 separate experiments. EGF did not stimulate incorporation of thymidine into DNA (Table II).

Experiments were conducted to verify that the ornithine decarboxylase activity in response to EGF was produced by keratinocytes. When EGF was added to confluent cultures of

3T3 cells, no ornithine decarboxylase activity was detectable. Similarly, 3T3 cells irradiated and plated at the density used for keratinocyte culture had no detectable ornithine decarboxylase activity with or without EGF. In contrast, bovine keratinocytes plated at high density in the absence of 3T3 cells and grown to confluent, stratified cultures responded to EGF (+ vehicle: 229 pmol mg^{-1} hr^{-1}, + EGF: 492 pmol mg^{-1} hr^{-1}).

Addition of cycloheximide to the medium abrogated ornithine decarboxylase activity (Fig 5). When added under similar conditions, actinomycin D did not affect ornithine decarboxylase activity in cultures not stimulated by EGF; however, the maximal increase in ornithine decarboxylase activity produced by EGF was slightly decreased by actinomycin D (Fig 5). Actinomycin D obliterated the prolonged elevation of ornithine decarboxylase activity caused by exposure to EGF (Fig 6).

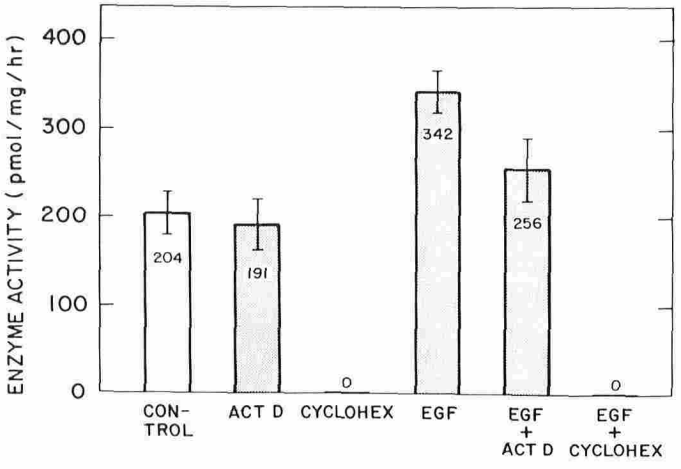

FIG 5. Effect of cycloheximide and actinomycin D on the response of ornithine decarboxylase activity to EGF. Twenty-three hours after DMEM change, cycloheximide (final concentration 5 μg/ml added in 25 μl PBS) or actinomycin D (final concentration 0.5 μg/ml added in 12.5 μl PBS:ethanol, 1:1) was added to bovine keratinocyte cultures. One hour later EGF or vehicle was added. Cultures were harvested 10.5 h after the addition of EGF and ornithine decarboxylase activity was determined as described in *Materials and Methods*. Each point represents the average of 2 dishes (bar ± SE), each assayed in duplicate.

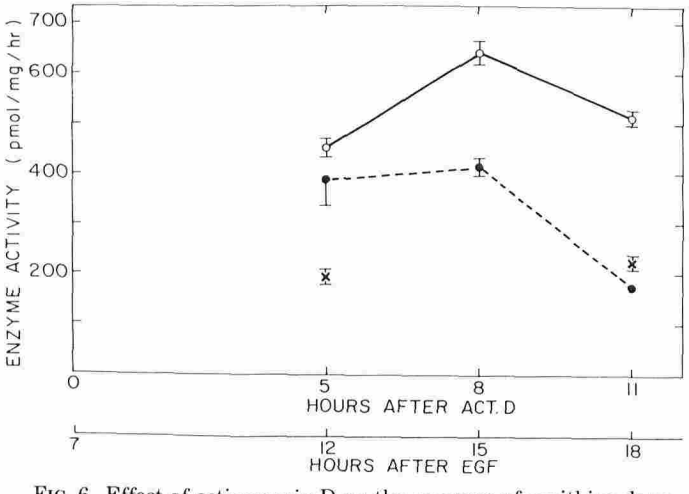

FIG 6. Effect of actinomycin D on the response of ornithine decarboxylase activity to EGF—time course. Twenty-four hours after the addition of serum-free DMEM, EGF or vehicle was added to bovine keratinocyte cultures. Seven hours later (31 h after DMEM change), actinomycin D (final concentration 2 μg/ml added in 50 μl ethanol) or ethanol (50 μl) was added. Cultures were harvested 12, 15, and 18 h after the addition of EGF or vehicle and ornithine decarboxylase activity was determined as described in *Materials and Methods*. Each point represents the mean ± SE of 2 cultures, each assayed in duplicate. ●-EGF + actinomycin D, O-EGF + ethanol, x-vehicle + ethanol.

DISCUSSION

In the work presented here we have determined the optimum conditions for assay of ornithine decarboxylase activity in keratinocytes and identified changes in keratinocyte ornithine decarboxylase activity in response to EGF and to serum-free medium.

Ornithine decarboxylase activity was maximal in MES buffer, pH 6.3, in the presence of EDTA, dithiothreitol, and pyridoxal phosphate. Purified calf liver ornithine decarboxylase exhibits optimal activity under similar buffer and pH conditions [21]. Keratinocyte ornithine decarboxylase activity was lower at more alkaline pH and in other buffers, conditions under which ornithine decarboxylase activity has usually been measured in skin [2,7,22]. It is important to optimize the conditions under which enzyme activity is determined in order to accurately determine initial reaction rate [23] and to detect low levels of enzyme activity.

EGF caused a concentration-dependent increase in keratinocyte ornithine decarboxylase activity (Fig 3). Although most experiments were conducted with bovine keratinocytes, similar changes were seen in human keratinocytes. It is unlikely that the response of ornithine decarboxylase activity to EGF is due to remaining 3T3 cells, as confluent cultures of 3T3 cells showed no such response and identical increases were seen in confluent cultures of epithelial cells plated at high density without a 3T3 feeder layer.

Under the conditions studied we saw no increase in [^{3}H] thymidine incorporation into DNA in response to EGF in the cultures 24 h after exposure to EGF. Although ornithine decarboxylase increase and DNA synthesis is often linked [24], an increase in ornithine decarboxylase activity without mitogenic response has been seen in mouse fibroblasts exposed to a cyanogen bromide cleavage product of EGF [25]. Taketani et al [26] recently reported a mitogenic effect 5 days after the addition of EGF to primary mouse mammary epithelial cell cultures. Our inability to detect an increase in labeled thymidine incorporation into DNA may reflect a dissociation of the increase in ornithine decarboxylase activity from mitogenesis. Alternatively, a mitogenic effect may be present, but detectable only several days after addition of EGF.

The magnitude of the increase in ornithine decarboxylase activity produced by EGF is small (2- to 3-fold) compared to that seen in response to medium change (6- to 8-fold) (Figs 2, 4). All experiments were conducted in serum-free medium. When we added EGF at the initial serum-free DMEM feeding we saw a slightly greater increase in ornithine decarboxylase activity than with DMEM alone (data not shown). By waiting 24 h after changing to serum-free DMEM we were able to detect a much greater response to EGF. Chen and Canellakis [27] found similar changes in a neuroblastoma cell line with serum-free DMEM vs asparagine as a stimulus.

The time course of increased ornithine decarboxylase activity subsequent to EGF exposure that we observed in cultured keratinocytes is that of a delayed and prolonged increase in activity. Similar patterns of ornithine decarboxylase activity have been seen in neuroblastoma cells exposed to asparagine [27] and in HTC (rat hepatoma cells) exposed to dexamethasone [28]. Our results differ in that we have observed a reproducible twin peak of ornithine decarboxylase activity in response to EGF (Fig 4). This type of biphasic increase in ornithine decarboxylase activity has been reported in rat liver after partial hepatectomy [29] and after subcutaneous injection with a hormone-containing solution or by shifting animals from a protein-free to a protein-containing diet [30]. In contrast, the increase in ornithine decarboxylase activity in mouse skin exposed to TPA or to EGF shows a peak 4–6 h after exposure followed by a rapid decrease in activity, similar to the changes in ornithine decarboxylase activity seen in our cultures after replacing serum-free DMEM. The prolonged response to EGF in vitro in comparison to in vivo might reflect one or more of

several events: altered binding of EGF to receptors or processing of EGF-receptor complexes by the cultured cells, altered synthesis or degradation of ornithine decarboxylase, altered synthesis or degradation of ornithine decarboxylase antizyme [31], or altered interaction of ornithine decarboxylase with its antizyme. EGF receptors have been demonstrated in cultured human foreskin epithelial cells [32]. We have not investigated the binding of EGF to receptor or processing of EGF-receptor complexes in our cultures. We know that ornithine decarboxylase activity is lost in the presence of cycloheximide (Fig 5). These findings suggest that protein synthesis is necessary to see enzyme activity. The prolonged elevation of ornithine decarboxylase activity is reduced by actinomycin D (Fig 6), suggesting that the early phase of increase in ornithine decarboxylase activity is independent of RNA synthesis while the later phase requires transcription. Definitive characterization of the synthesis or degradation of ornithine decarboxylase or ornithine decarboxylase antizyme or of their interaction awaits improved methods for studying both molecules [33].

The response of cultured normal epithelial cells to EGF suggests that the response of whole skin to EGF in vivo and in vitro may not be entirely due to the effects of EGF on dermal cells [34], but may in addition be related to the interaction of EGF with epidermal cells. Characterization of the early biochemical changes in normal epithelial cells exposed to EGF may lead to better understanding of the mechanism of action of EGF on the epidermis.

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